Design and Evolution of Biocatalysts

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Abstract: Enzymes as biocatalysts offer several advantages over their chemical counterparts, and as such have attracted much attention for use in the synthesis of various organic compounds. However, despite many successes in the practical application of enzymes, the extensive use of enzymes in the synthesis of organic compounds is still hindered by inadequacy in substrate specificity, catalytic activity, enantio-selectivity and stability. Enzymes with desired functions targeted for practical applications have long been a goal in protein/enzyme engineering. Many approaches have been developed and employed for redesigning enzymes with desired properties, including the structure-guided rational method, directed evolution, computational methods, and combinatorial methods. This review will cover recent advances in the design and evolution of enzymes targeted for specific properties, focusing on the strategy and the applicability of each approach.

Key Words: Biocatalyst, rational design, directed evolution and computational design.

1. INTRODUCTION

Enzymes are currently used in many areas since their enantiomeric specificity is suitable for the production of optically pure compounds as well as bulk chemicals. However, despite the many advances in enzyme-based processes, the practical applications of enzymes have been limited by their insufficient substrate specificity, low thermal stability, and low tolerance in organic solvents. Success of enzyme-based processes mainly relies on the properties of the enzymes, and some innate drawbacks of enzymes including stability, catalytic efficiency, and enantioselectivity should be significantly improved to meet the requirements for these enzyme-based processes. In an effort to broaden the use of enzymes, a number of approaches have been developed to endow them with desired properties including the structure-guided rational design, directed evolution, and computational methods. These combined efforts have resulted in marked advances in understanding and evolving enzymes.

Directed evolution has become a general tool used to create targeted enzymes without knowing the 3-D structure of the protein; however, this method requires a high throughput screening system. With increased knowledge on the structural and mechanistic properties of an enzyme, rational and computational methods become more accurate tools for engineering enzymes and these methods have culminated in several notable successes. As more knowledge about the structure-function relationship of enzymes becomes available, the easier it is to design enzymes. These two approaches are usually combined to provide complementary effects that essentially compensate the limitations of each individual approach. Recently, issues on global warming and sustainable economic growth has resulted in increased attention on the implementation of enzyme-based green processes, further promoting the design of enzymes with desired properties. In the present review, we will introduce recent advances in the design and evolution of enzymes targeted for specific properties, focusing on the strategy and the applicability of each approach.

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2. METHODS FOR ENZYME DESIGN

Several methods to generate enzymes with improved properties have been developed, and they can be categorized into structure-based rational design, library-based random approach, and combinatorial method. Rational design is based on the assumption that the structure-function relationship can be predicted by the use of mechanistic knowledge. The predictions are tested by site-directed mutagenesis or saturation mutagenesis at the designed position. Random design deals with subtle unpredictable effects at distant positions. Amino acid residues distant from the active site of an enzyme affect the properties of the protein through the hydrogen bond network, stability, flexibility, etc. Random mutations are created by error-prone PCR and DNA shuffling and the application of random design requires an appropriate screening system. The combinatorial method is a combination of both rational and random approaches, which includes using a focused library in the designed area, saturation mutagenesis of the library-screened mutant position, and performing the random approach from a designed starting mutant.

A few variations of these basic techniques include loop remodeling, *in silico* mutagenesis and multiple saturation mutagenesis [1-4]. *In silico* mutagenesis is based on computation simulations of the mutagenesis effect through energy evaluation and requires experimental confirmation [3, 5]. Loop remodeling uses a substitution of a whole loop structure rather than a mutation of a single amino acid residue. The combinatorial active site saturation test (CAST) utilizes a strategic combination of saturation mutations at the active site to control the size of the library while guaranteeing all the combinations occur, which are usually not available in the error-prone PCR library [4].

Many variations of these techniques are reviewed in detail elsewhere [6, 7]. As structural and mechanistic knowledge about enzymes increases and structures become more readily available, rational design approaches will be used more often. In addition, since computation capacity is rapidly increasing, the use of *in silico* screening and computer-assisted combinatorial design will also increase.

3. TARGETED FUNCTIONS OF ENZYME FOR REDESIGNING

In order for enzymes to be practically implemented for the production of valuable compounds, their properties need to be im-

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proved. The properties that typically require improvement are catalytic activity, substrate specificity, stability, enantioselectivity, and expression levels. Enzymes should have higher catalytic activity for greater productivity, and often require new catalytic power to catalyze non-natural substrates. For the synthesis of chiral compounds, high enantioselectivity of the enzyme is a prerequisite. In addition, enzymes are required to maintain their activity during a reaction under specific reaction conditions (temperature, pH, and organic solvents). The stability of enzymes is considered one of the weakest points of biocatalysis compared to chemical catalysis. Isolation of thermostable enzymes, modification or immobilization of enzymes, and stabilization of enzymes by rational design and directed evolution have been attempted to overcome these drawbacks. The engineering of enzyme stability is reviewed in other papers [8, 9]. We will mainly focus on the progress made in the chemical reaction-related properties of enzymes.

3.1. Catalytic Activity

Natural enzymes are believed to have evolved from a progenitor scaffold through a number of steps including duplication and rearrangement of relevant genes. It is generally accepted that the promiscuous activity of ancestor enzymes played a significant role in the generation of currently existing enzymes [10]. Enzymes with promiscuous activity are known to display wide substrate specificity for their target substrate. They have been rarely modified to improve catalytic activity for their major substrate, but promiscuous activity for their minor substrate can be easily enhanced without significantly affecting the activity for major substrate.

Improving the catalytic activity of an enzyme to the same level it had for its original substrate is uncommon and difficult to achieve since the enzyme has already evolved specifically for a certain substrate; the catalytic amino acids in the active site are optimally orientated, and the binding pocket is well-established. Thus, the only possible method to achieve this would be random mutagenesis, and there are only a limited number of reports where this method has been successfully implemented. Xylanase A was evolved to have higher activity at extreme pHs to help bleaching of pulp [11, 12]. A mutant (2TfxA98) with five mutations was found after two rounds of DNA shuffling using a pH 9.0 agar plate. The mutant showed a 12-fold higher k_{cat}/K_M value than the wild-type enzyme, increasing from 48 s^{-1} $(mg/ml)^{-1}$ to 562 s^{-1} $(mg/ml)^{-1}$.

To increase the catalytic efficiency of epothilone-B hydroxylase, the effects of various mutations located at proximal and distant sites were investigated [13]. Diverse variants were generated by site-directed and random mutagenesis based on combinations of 15 amino acid residues. Of the 15 sites, five residues near the catalytic site affected the shape and the size of the binding pocket and consequently the catalytic efficiency. Based on a comparison of the structures of homologous proteins in their substrate-bound and free forms, five loops were suggested to be the most dynamic part in catalysis. Most distal mutations found near these parts of the enzyme affected the opening of the lid structure upon substrate binding. Destruction of a specific salt bridge and disruption of long-range coupled-interactions also had an effect on the opening of binding pocket, causing changes in the pocket size and shape. This analysis provided a general guideline for the systematic investigation of the effect of mutations on catalysis.

3.2. Substrate Specificity

Studies on increasing the catalytic activity of enzymes for their major substrate are rare, but there have been many cases where the specific activity of the enzyme for minor substrates was improved. A small change in the substrate binding pocket can increase its activity toward analogous substrates. Many reviews regarding expanding the substrate specificity of the family enzymes have already been published [14-16]. Moreover, changing the substrate specificity is often required for practical applications. When the synthesis of non-natural compounds is possible only through the use of naturally occurring enzyme, the enzyme is usually employed despite its low substrate affinity. In an attempt to increase the productivity of target compounds from non-natural substrates, enzymes are modified for higher substrate specificity.

Mandelamide hydrolase (MAH) from *Pseudomonas putida* has wide substrate specificity for various lactamide substrates shown in Fig. (**1**) with a strong preference for aromatic lactamides, which is a member of the amidase family [17]. Amidases have broad industrial applications including the synthesis of chiral compounds [18]. After two rounds directed evolution using error-prone PCR, the specificity of the enzyme changed to aliphatic lactamides, resulting in a decreased K_M value. Two amino acids at the active sites were suggested to be involved in determining enantio-selectivity and aromatic group recognition. In many cases, only a small change in the active site can increase the activity of the promiscuous enzymes for weak-activity substrates [19-22]. Thus, site-directed mutagenesis and saturation mutagenesis of active site amino acid residues have been frequently employed to enhance the substrate specificity of a given enzyme.

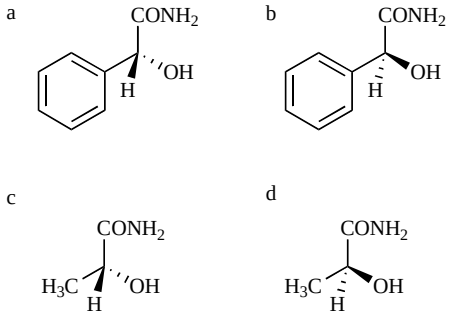

Fig. (1). Substrates of mandelamide hydrolase. (**a**) and (**b**) Isomers of mandelamide. (**c**) and (**d**): Isomers of lactamide.

Xylose reductase from *Candida tenuis* (CtXR) shows broad substrate specificity toward various α-ketoesters shown in Fig. (**2**). CtXR produces α-hydroxy esters, which are essential compounds in

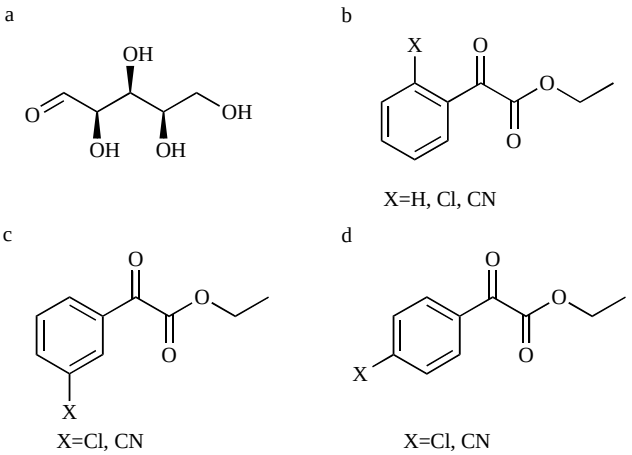

Fig. (2). Substrates of xylose reductase. (**a**) Xylose ; (**b**), (**c**) and (**d**) Aromatic α-keto esters.

various organic syntheses. Docking simulation of CtXR with xylose suggested Trp23 was the key residue in substrate binding [23]. The W23F and W23Y mutants generated by site-directed mutagenesis showed up to 14,000-fold higher substrate specificity for aromatic α-ketoesters over xylose. The increase was revealed to result from both the reduction in xylose efficiency and the increase in α-ketoesters efficiency.

Monoamine oxidase from *Aspergillus niger* (MAO-N) was engineered to have broader substrate specificity toward secondary and tertiary amines [24]. Mutant enzymes generated by directed evolution were extensively analyzed to provide further insight into the structure-function relationship, and the effect of each mutation on the substrate specificity was investigated. A hypothesis was suggested: two mutations reduced the steric hindrance behind the "aromatic cage" at the active site, conferring flexibility upon substrate binding; Two other mutations remotely influenced the orientation of the Phe382 side chain and consequently the electrochemical and steric environment near the FAD binding site.

Phenylalanine dehydrogenase (PheDH), which acts on non-natural amino acids, was developed through EP-PCR, a combination of mutations, and multi-site saturation mutagenesis [20]. PheDHs are used in phenylyketonuria diagnosis and production of enantiomerically pure non-natural amino acids [25, 26]. The catalytic efficiency, k_{cat}/K_M (s^{-1} mM^{-1}), for DL-propargylglycine increased by 7.1 fold (Fig. **3b**). This H7H10 mutant also showed an increased activity towards the original substrate L-phenylalanine, whereas the selectivity remained unchanged compared to the wild-type enzyme (Fig. **3a**). A combination of several positive mutations did not yield a better mutant. After additional saturation mutagenesis, the 25B12 mutant exhibited a 7.4-fold higher activity towards the target substrate and an 82-fold lower activity for the original substrate, resulting in a 612-fold higher selectivity. The catalytic efficiency of 25B12 for the new substrate (0.04 s^{-1} mM^{-1}) was lower than the wild-type enzyme for the original substrate (40.5 s^{-1} mM^{-1}) and even lower than the 25B12 mutant for the original substrate (0.49 s^{-1} mM^{-1}). In most cases of designing substrate specificity, the catalytic activity of the resulting enzyme was very low for the targeted substrate [2, 17], and increased selectivity mainly resulted from decreased activity towards the original substrate [22, 27]. Unknown remote mutations can cause changes in catalytic efficiency between the wild-type and designed enzymes. Structural analysis and precedent experiments can provide some insights into the major amino acid residues affecting the substrate specificity over entire regions of the enzyme rather than just a single amino acid residue, enabling the development of a focused library. Mutants in the first round of screening are used to generate a saturation mutagenesis library. Otherwise, mutations found from a library of random mutagenesis were combined with the site-directed mutagenesis [21, 28-30] and subjected to additional rounds of random mutations [31]. In some cases, major key residues were selected from the site-directed or saturation mutagenesis at the active site of the enzyme followed by further rounds of random mutagenesis to identify the remote mutation sites [32].

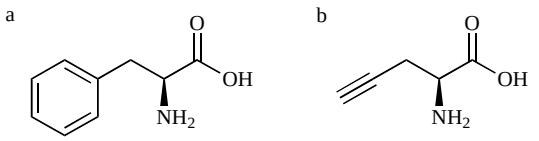

Fig. (3). Substrates of phenylalanine dehydrogenase. (**a**) L-phenylalanine (original substrate) ; (**b**) L-propargylglycine (non-natural amino acid).

The computational design method is useful when dealing with a designated region of an enzyme. The energy-based scoring function as well as structure modeling and docking programs provide crucial tools for virtual screening of mutants *in silico* over short periods of time, which are not possible using experimental approaches. Various methods for computational designing are available, and emerging technologies have been reviewed [33-35]. Computational methods can be utilized for designing enzymes, but their accuracy and applicability are not currently universal across a range of different enzymes. In many cases, computational calculations were used for analyzing engineered mutants rather than for predicting possible mutant designs [13, 22, 36-38]. Nonetheless, some successes in designing and engineering substrate specificity by computational methods have been reported. The loop structure of human guanine deaminase (hGDA) was designed to have higher specificity toward ammelide as an intermediate step in an effort to generate human-oriented cytosine deaminase (Fig. **4**) [2]. An important loop was designated by comparing homologous structures, and its structure and sequence were designed to have proper catalytic residues with a desired orientation by neighboring mutations. The redesigned enzyme showed a 100-fold higher activity towards ammelide. Even though the selectivity increased by about 2e6-fold, the specificity constant was still much lower than that of the wild-type enzyme toward guanine, its original substrate. More rational and improved methods remain to be developed for designing enzymes with catalytic activity that is comparable to the wild-type.

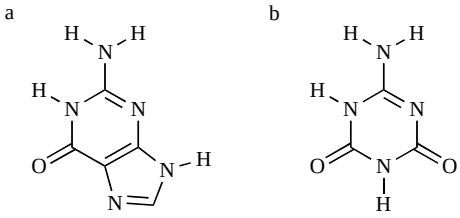

Fig. (4). Substrates of guanine deaminase. (**a**) Guanine; (**b**) Ammelide.

Simple computational methods have been widely employed to predict the structure of enzymes and to simulate the docking structure of the enzyme-substrate complex [22, 39, 40] as well as to analyze the resulting mutant enzymes [24, 41-44]. D-hydantoinase from *Bacillus stearothermophilus* SD1 (Hyd) has substrate specificity towards a small non-substituted hydantoin (Fig. **5a**). Hyd is commercially used in the synthesis of an intermediate like D-hydroxyphnylhydantoin (D-HPH) for various semi-synthetic antibiotics (Fig. **5b**) [45]. The enzyme's substrate specificity could be successfully modulated by manipulating the loop structure of active site [46]. Following this result, improved rational design was applied to design substrate specificity of the same enzyme. Comparisons of docking simulations between the target substrate D-HPH and original substrate hydantoin (hyd) revealed the major amino acid residues that determined the substrate specificity [22]. Mutations were designed based on the size and the hydrophobicity of the amino acid residues. Analysis of the resulting mutant confirmed the

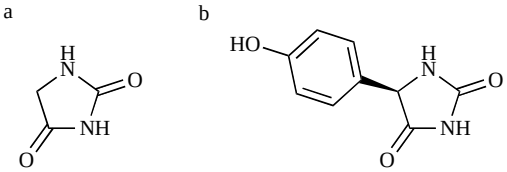

Fig. (5). Substrates of D-hydantoinase. (**a**) Hydantoin; (**b**) D-hydroxyphenylhydantoin (D-HPH).

specificity-determining factors, and the best mutant showed a 7-fold increased activity towards HPH. The selectivity of the designed mutant for HPH was about 200-fold higher than that of the wild-type enzyme. From additional computational analysis in terms of binding energy, the mutant enzyme was shown to bind more tightly with the transition-state substrate than the wild-type, which confirmed the utility of computational analysis.

Endo-chitosanase from *Bacillus circulans* MH-K1 (Csn) was engineered to create an exo-chitosanase that could produce pure chito-oligosaccharides [27]. Exo-chitosanase was designed by comparing the structures between endo-glucanase and exo-glucanase. Two loops in the binding cleft were designed, and more than 25 combinations were examined. Modeling of the structure revealed the best candidate with negligible backbone movement. The resulting mutant showed a significant difference in regards to the viscosity of the reaction mixture, which provided evidence for the exo-type cleavage. The activity was 3% of the wild-type enzyme, which implies that the specificity was acquired at the price of activity loss, as was previously observed.

Chitinase from *Beauveria bassiana* (Bbchit1) was engineered to have increased chitinolytic activity through three rounds of DNA shuffling [47]. As a result, two mutants showing an increased activity were found to possess two and four mutations, respectively. Interestingly, these mutation sites were located outside of the substrate binding and catalytic sites. From this result, it was inferred that an increase in the catalytic efficiency requires not only a designing catalytic site but also long-distance manipulations like backbone flexibility, domain motion, or the subunit arrangement. In another case, cyclodextrin glucanotransferase from *Thermoanaerobacterium thermosulfurigenes* (CGTase) was engineered to eliminate a side reaction [41]. Two variants displaying high cyclization activity over hydrolysis were selected after screening a random mutagenesis library, and each mutant contained a single mutation. Mutational analysis revealed that the loss of a hydrogen bond occurred in the S77P mutant and Tyr107 formed a new hydrogen bond with Arg228. This, in turn, retorted the orientation of Glu258, resulting in a loss of hydrolytic activity. For the other mutant W239R, the mutation disturbed a space filling and another hydrogen bond.

Expansion of substrate specificity has been attempted for either superfamily or evolutionarily-related enzymes. In this case, even a single mutation or a few site-directed mutagenesis enabled interconversion of two enzymes [48]. A few transferases and their related hydrolases were reported to be easily inter-converted because they share the same mechanism of intermediate formation [29, 48-50]. If water acts as the acceptor, the enzyme catalyzes the hydrolysis reaction. On the other hand, the enzyme transforms into a transferase in the presence of a different acceptor. Cyclodextrin glucanotransferase (CGTase) was engineered to α-amylase through saturation mutagenesis around the active site of the enzyme [51]. While the wild-type enzyme had a low hydrolytic activity, a mutant with three mutations had an 11-fold higher k_{cat} for hydrolysis and 350-fold lower k_{cat} for glucanotransferase activity, resulting in 4000-fold increase in the hydrolysis reaction compared to glucanotransferase activity. The k_{cat} value for hydrolysis was one-third of the natural amylases. A separate error-prone PCR library yielded mutants showing much lower activity. This result demonstrates the importance of a focused library, and a random mutation library alone is unlikely to be enough to search for desired variants.

A lipase from *Pseudomonas aeruginosa* was converted to an amidase by directed evolution [52]. The two enzymes share the same serine hydrolase mechanism as well as acyl-enzyme intermediate. The best mutant from a random mutation library showed a 13-fold increased activity towards the amide substrate than that of the wild-type enzyme. Another mutant generated through saturation mutagenesis of the active site displayed a 28-fold increase in activity. The esterase activity of both enzymes was as high as the wild-type enzyme. Based on the structure of the mutants, a hot spot for amidase reaction was suggested and a reaction mechanism was proposed.

β-Galactosidase from *Escherichia coli* was converted to β-glucuronidase by directed evolution [53]. Mutant enzymes were screened for their activity toward p-nitrophenyl-β-D-glucuronide (pNPG) and o-nitrophenyl-β-D-galactopyranoside (oNPG). The best mutant showed a 3-fold increased activity toward pNPG while retaining 70% activity toward oNPG. The catalytic activity for oNPG (144 U/mg) was found to be higher than that for pNPG (3.4 U/mg). In the conversion from hydrolysis to transglycosylation in β-glucosidase from *Themotoga neopolitana*, the activity for both reactions decreased [49].

Firefly luciferase is a bi-functional enzyme that has both monooxygenase and fatty acyl-CoA synthetase (FACS) activities. This enzyme was presumed to have evolved from FACS. The site-directed mutagenesis of FACS at the conserved serine residue resulted in the generation of luminescence activity [54]. The luminescence unit was marginally better than background level, but much lower than the natural luciferase. Nevertheless, this result indicates an important step and the hot spot for divergent evolution from FACS to luciferase.

3.3. *De novo* Catalytic Activity

Designing enzymes with *de novo* activity remains a challenging issue in protein engineering. To introduce a new catalytic activity into a pre-existing protein scaffold, many factors should be taken into account including substrate binding, metal coordination, catalysis, etc. To achieve this goal, many methods have been attempted based on rational and random approaches as well as computational methods.

A notable success in the evolution of an enzyme with new catalytic activity was shown for glyoxalase II (GlyII). Through combinatorial approaches, including rational design and directed evolution, GlyII was successfully converted to metallo β-lactamase (MBL) [1]. GlyII has no activity at all towards cefotaxime, which is a substrate for MBL (Fig. **6**). Thus, to endow Glyll with a new catalytic activity for cefotaxime, a completely different approach was adopted from those most often used for designing the substrate specificity of enzymes with low activity. To generate the *de novo* activity starting from the existing scaffold protein, several critical loops were randomly organized followed by incorporation into appropriate positions in the enzyme using the SIAFE (simultaneous incorporation and adjustment of functional elements) process. The evolved metallo β-lactamase 8 (evMBL8) completely lost its original activity for S-D-lactoylglutathione but attained β-lactamase activity, which had a k_{cat}/K_M value of 1.8e2 M^{-1} s^{-1} for cefotaxime. This work provided evidences for divergent evolution in different protein family enzymes.

A retro-aldol enzyme acting on the non-natural substrate, 4-hydroxy-4-(6-methoxy-2-naphthyl)-2-butanone (Fig. **7**), was created using a computational method [3]. The method includes selecting a scaffold protein, computing the transition state for a functional group, positioning the functional group in the active site, optimizing

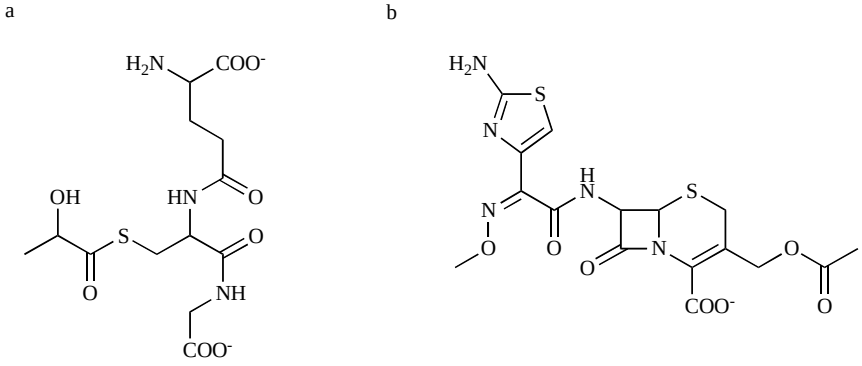

Fig. (6). (**a**) S-D-Lactoylglutathione (substrate of glyoxalase II); (**b**) Cefotaxime (substrate of metallo-β-lactamase).

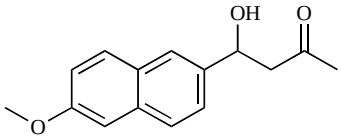

Fig. (7). Substrate for retro-aldol reaction, 4-hydroxy-4-(6-methoxy-2-naphthyl)-2-butanone.

adjacent amino acids, and estimating the binding energy. Of 72 candidates, 32 showed detectable retro-aldolase activity. The catalytic efficiency of the designed mutants ranged from 0.016 to 0.74 M^{-1} s^{-1}, and the k_{cat}/k_{uncat} reached 10^3 to 10^4. Increasing the catalytic efficiency of the mutant enzyme to the level of the natural enzyme has not yet been achieved and is one of the goals of future studies.

An enzyme with Kemp elimination reaction was designed using another computational method [5]. No known natural biocatalyst catalyzes this reaction (Fig. **8a**). Thus, the catalytic base amino acid for the reaction was designed, and then additional mutations for stabilizing the transition state structure were predicted. Enzymes with different template structures, catalytic bases, and hydrogen donors were generated. The resulting designs showed k_{cat}/K_M values up to 78 M^{-1} s^{-1} towards target substrate, 5-nitrobenzisoxazole (Fig. **8b**). Seven rounds of additional directed evolution resulted in an enzyme with 200-fold increased catalytic efficiency. Based on this work, computational design of functional residues and further evolutionary screening of evolved enzymes using directed evolution seems to be an excellent combination for the design of new enzymes.

3.4. Enantio-Selectivity

Biocatalysis is preferred over chemical reactions mainly because of the enantioselectivity of enzymes. The enantioselectivity of enzymes is largely determined by the structure of the substrate binding pocket, and other subtle changes also affect the selectivity to some extent.

Baeyer-Villiger monooxygenase (BVMO) from *Pseudomonas fluorescens* SDM 50106 has moderate enantiomeric excess (E) of around 55 for aliphatic acyclic ketones, which makes (S)-hydroxyalkylacetate useful for the synthesis of aliphatic (S)-1,2-diols. The E value for 4-hydroxy-2-ketones was increased up to 92 by a round of random mutagenesis using error-prone PCR and additional combination of the resulting mutations [28]. However, as the enantioselectivity increased, the conversion yield linearly decreased. This is similar to acquiring substrate selectivity at the cost of reduced activity towards the original substrate [27].

Esterase from *Burkholderia gladioli* (EstB) exhibits moderate S-enantiospecificity (E_S = 6.1) towards methyl-β-hydroxyisobutyrate (Fig. **9**). A random mutation library was used to show that position 152 was an important residue in determining enantioselectivity [55]. Nineteen amino acid residues at the active site were changed by saturation mutagenesis and best variant, with E_R=28.9, was generated. The activity was also retained at a level equal to the wild-type enzyme, reaching about 3 U mg^{-1}. These design steps are a typical example that shows the potential of current design techniques. Once the initial mutation experiment revealed important residues, saturation mutagenesis and random mutation were used.

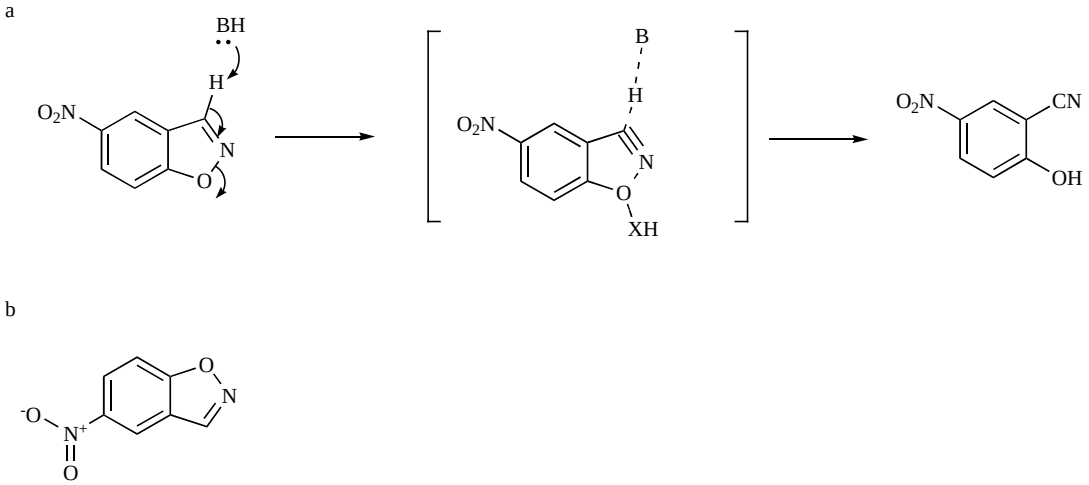

Fig. (8). (**a**) Kemp elimination reaction scheme ; (**b**) 4-Nitrobenzisoxazole (a kemp elimination substrate).

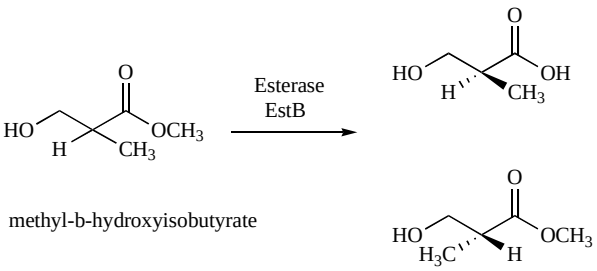

Fig. (9). Enzymatic kinetic resolution of methyl-β-hydroxyisobutyrate catalyzed by esterase.

Mutations of selected variants were either combined or further confirmed through a combination of saturation mutagenesis. The resulting mutant was analyzed in terms of mutation sites and used for another round of evolution. In the case of Arylmalonate decarboxylase, site-directed mutagenesis of the enzyme resulted in inversion of enantioselectivity toward either α-naphthyl-α-methylmalonate or α-thienyl-α-methylmalonate, but the catalytic activity decreased (Fig. **10**) [56]. The lowered catalytic activity was recovered up to 10% of the wild-type enzyme by several rounds of random mutations.

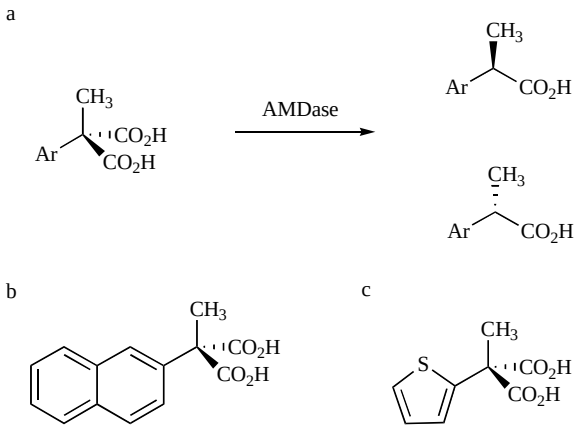

Fig. (10). Enantioselective decarboxylation. (**a**) Reaction scheme; (**b**) α-Naphthyl-α-methylmalonate ; (**c**) α-Thienyl-α-methylmalonate.

Enantioselectivity of esterase from *Pseudomonas fluorescens* (PFE) was altered by random mutagenesis, but the resulting mutants were expressed as an inclusion body with very low activity [57]. The effect of each mutation on the enantioselectivity was studied to predict which residues affected the enantioselectivity. By inverting some mutations at the active site, the activity and enantioselectivity were increased. Similarly, lipase was subjected to random and site-directed mutagenesis to enhance the enantioselectivity. Analysis of the mutant revealed that one residue was located at the active site and three were found in a remote site [42]. Based on the results, it is likely that the enantioselectivity of enzyme was changed by alterations in the amino acid residues near the active site as well as alterations in the remote amino acid residues, which makes it difficult to design the enantioselectivity of an enzyme using only a rational approach.

3.5. Cofactor Specificity

Many enzymes require various kinds of cofactors (coenzymes) for their reactions. In particular, NADH and NADPH are major cofactors in electron transfer-mediated enzymes. From a practical standpoint, the development of cofactor regeneration systems is crucial for decreasing the cost of enzyme processes [58]. In addition, engineering the coenzyme specificity of an enzyme [59] such that inexpensive cofactors can replace expensive cofactors will be vital to expanding the use of enzyme-based biocatalysis for commercial production of organic compounds. Many attempts have been made to alter cofactor specificity using various approaches. Basically, the design of coenzyme specificity seems to be similar to that of substrate specificity in terms of binding and reaction mechanisms.

A short chain carbonyl reductase (SCR) from *Candida parapsilosis* is known to show a strict coenzyme preference for NADPH. To change the specificity toward NADH, amino acid residues in the coenzyme binding pocket were mutated, and NADH-utilizing mutants were screened to identify a mutant displaying a complete loss of activity for NADPH [59]. Xylose reductase from *Pichia stipitis* also exhibits coenzyme specificity towards NADPH over NADH. By comparing the binding structures of the enzyme with NADPH and NADH, Lys21 was chosen as a key residue because only this residue resulted in a difference in coenzyme binding [54]. Site-directed mutagenesis generated a mutant enzyme with no activity in the presence of NADPH, while the enzyme activity with NADH remained at a similar level as the wild-type. The specific activity of the mutant enzyme with NADH was found to be one fourth the activity of the wild-type enzyme in the presence of NADPH.

Formate dehydrogenase from *Candida boidinii* (CboFDH) has a strong cofactor preference for NADH over NADPH. Based on a comparison between sequences and structures of homologous enzymes, target amino acid residues were selected to change the cofactor specificity [60]. The first round of saturation mutagenesis on the target residue created a mutant enzyme with 10^5-fold increased k_{cat}/K_M in the presence of NADPH over that of the wild-type enzyme. Additional saturation mutagenesis at neighboring residues further increased the k_{cat}/K_M by 40-fold for NADPH. When considering the specific constant of the enzyme in the NAD^+-mediated reaction, an increase in the coenzyme preference ranged from 10^7 to 10^8 folds. The resulting CboFDH variant is expected to be used for NADPH regeneration in conjunction with cytochrome P450 monooxygenase.

4. CONCLUSION

Demand for a bio-based economy increasingly requires broadening the use of biocatalysts for the industrial production of different chemical products. To achieve this, design and evolution of enzymes with desired properties are a prerequisite. Significant achievements have recently been made in this regard due to the advances in relevant technologies. With recent progress in systems biology and the accumulation of genome sequences, designing enzymes is also expected to play a crucial role in metabolic engineering and synthetic biology. However, to more readily achieve these goals, some key issues should be addressed including the development of high throughput screening systems, library construction, understanding of structure-function relationships of proteins, mechanistic knowledge on enzymes, and accurate modeling of protein structure. Depending on the current knowledge and availability of related techniques, an appropriate approach should be chosen and implemented for the successful design of enzyme with targeted function.

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